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Date: February 1, 1995

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Re: Final Technical Report for N00014-91-J-1231

Summary of accomplishments: Please find attached a manuscript, recently accepted by *Science*, which provides an excellent summary of the work accomplished through the above referenced grant. This is the work outlined in our last progress report submitted in June, 1994.

Personnel supported: The funding from this grant helped support the work of the postdocs Nancy Lim, Xian-cheng Yang, Mark Nowak, and Patrick Kearney. It also helped support the work of Cesar Labarca (a senior scientist), and Scott Silverman and Wenge Zhong (graduate students).

Significance: The incorporation of unnatural amino acids will eventually allow researchers to tailor proteins for the deisred characteristics. The single-channel resolution of the patch-clamp technique provides an accurate assessment of the properties of the modified proteins.

Publications: Please see attached paper in press in *Science* by Nowak et al.

Nicotinic Receptor Binding Site Probed with Unnatural Amino Acid Incorporation in Intact Cells

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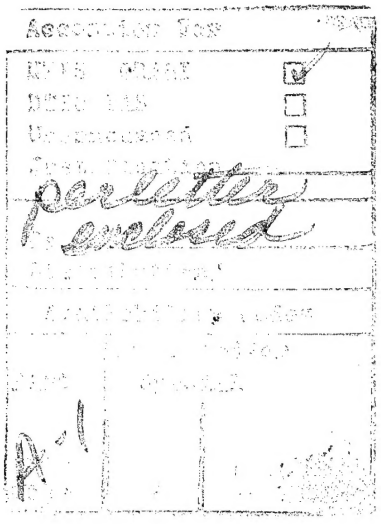

The nonsense codon suppression method for unnatural amino acid incorporation has been applied to intact cells and combined with electrophysiological analysis to probe structure-function relationships in the nicotinic acetylcholine receptor. Functional receptors were expressed in *Xenopus* oocytes when tyrosine and phenylalanine derivatives were incorporated at positions 93, 190, and 198 in the binding site of the α subunit. Subtle changes in the structure of an individual side chain produced readily detectable changes in the function of this large channel protein. At each position, distinct features of side-chain structure dominate the dose-response relation, probably by governing the agonist-receptor binding.

In the study of membrane-bound receptor, channel, and transporter proteins, classical pharmacology has defined highly specific agonists and antagonists; and quantitative structure-activity studies have generated many hypotheses concerning ligand-receptor interactions. More recently, the combination of site-directed mutagenesis and heterologous expression has enabled functional studies on the consequences of structural modifications of the receptors. In the absence of atomic-scale structural data for membrane-bound receptors, these methods provide detailed information for studying ligand-receptor interactions. First generation mutagenesis methodologies employed the normal translation machinery such that a residue of interest could be changed to any of the other 19 natural amino acids.

Site-directed mutagenesis combined with nonsense suppressors--tRNAs altered at the anticodon so that they insert an amino acid in response to an mRNA termination codon--have allowed the generation of several proteins with known amino acid changes from a single mRNA[1]. Recently, second generation mutagenesis methodologies incorporated the nonsense suppression principle and extended the amino acid repertoire by providing a means for the site-specific incorporation of unnatural amino acids into proteins in cell-free systems[2,3]. We report here the adaptation of this approach to a heterologous expression system in an intact cell. Combined with the high sensitivity and resolution of modern electrophysiological techniques, the incorporation of unnatural amino acids provides a general method for structure-function studies of receptors, channels, and transporters.

Figure 1A outlines the nonsense codon/tRNA suppressor method as adapted to intact eukaryotic cells. A *Xenopus* oocyte is coinjected with 2 mutated RNA species: 1) mRNA, synthesized *in vitro* from a mutated cDNA clone containing a stop codon, TAG, at the amino acid position of interest; and 2) a suppressor $tRNA^4$ containing (a) the complementary anticodon sequence (CUA) and (b) the desired unnatural amino acid synthetically acylated to the 3' end[5,6]. During translation by the oocyte's synthetic machinery, the unnatural amino acid is specifically incorporated at the appropriate position in the protein encoded by the mRNA.

We have exploited this method to study a ligand-gated ion channel, the nicotinic acetylcholine (ACh) receptor[7]. The muscle-type ACh receptor (AChR) contains five subunits with a stoichiometry of $\alpha_2\beta\gamma\delta$. An appropriate subject for this first investigation is the interaction between ligands and the amino acid residues in the α subunit at positions 93, 190 and 198. There is good evidence (though not conclusive proof) that these residues contribute to the agonist binding pocket[8]. The wild type residue is tyrosine at each of these positions in nearly all known AChR α subunits[9]. Previous studies show that phenylalanine at these positions modifies the binding of ACh, with minimal effects on the subsequent conformational change that opens the channel[10]. The binding of d-tubocurarine (dTC) and several other competitive antagonists is also affected by mutations at these positions[11]. We have examined several measures of receptor function as affected by the incorporation of various tyrosine and phenylalanine derivatives (Figure 2) at positions 93, 190 and 198.

A major concern from the outset was possible reacylation of the injected suppressor tRNA with natural amino acids by endogenous aminoacyl-tRNA synthetases. Reacylation could occur after delivery of the non-natural amino acid to the ribosome or via a synthetase editing mechanism[12], which removes the unnatural amino acid and replaces it with a natural one. Either type of reacylation would lead to an uncontrollable mixture of amino acids at the site of interest in the protein. Therefore, a tRNA amber suppressor (anticodon CUA; tRNA-MN3) having a low probability of reacylation by each of the 20 synthetases in the cell (Figure 1B) was designed by altering a yeast $tRNA^{Phe}_{CUA}$ used previously to incorporate unnatural amino acids in an *in vitro* translation system[2]. Two changes made previously in yeast $tRNA^{Phe}$ (G34C, A35U) introduced the amber anticodon and removed known recognition elements for eukaryotic phenylalanyl-tRNA synthetases (PheRS)[13]. However, these changes also introduced a recognition site for TyrRS and for GlnRS. Because the wild type C2-G71 base pair could also contribute to recognition by TyrRS and GlnRS[14], this base pair was changed to A-U in tRNA-MN3. In addition, the G20A mutation was introduced to decrease reacylation by PheRS. Like the original yeast $tRNA^{Phe}_{CUA}$, tRNA-MN3 also contains the G37A mutation to increase suppression efficiency[15].

We first estimated, using in vitro translation of the AChR α subunit in a reticulocyte lysate system[16], that the incorporation efficiency is on the order of 10% for several unnatural amino acids[17,18]. If this efficiency applies to translation in intact *Xenopus* oocytes as well[19,20], one would expect generation of full-length α subunits to be

rate-limiting for the production of oligomeric receptors. Because two α subunits are incorporated into each complex, 10-100-fold greater amounts of α subunit mRNA, relative to β, γ, and δ subunit mRNAs, were injected.

To test the efficacy and fidelity of unnatural amino acid mutagenesis in oocytes, we incorporated into the α subunit (a) the natural tyrosine residue at position 190, (b) the natural tyrosine at 198, or (c) a phenylalanine at 198. Currents of tens to hundreds of nA were observed at -80 mV as soon as 6 h post-injection, and currents generally greater than 1 μA were observed 24 h after injection. Electrophysiological properties of the expressed receptors were identical to those of the wild-type (Figure 3A, B) or the previously studied αTyr198Phe mutant generated by conventional mutagenesis (Figure 3D). Thus, the method yields the expected data when compared with conventional mutagenesis and heterologous expression. There were no responses when no tRNA at all was injected.

The most important control experiment involved coinjection of the full-length but unacylated tRNA-MN3 with the mRNA, because oocytes can acylate injected tRNAs[21]. In 19 out of 20 experiments, each involving 5-10 oocytes and typified by Fig. 3C, no response at concentrations as high as 800 μM ACh was seen up to 24 h after injection. In contrast, after injection of the unacylated $tRNA^{Phe}_{CUA}$, there were detectable responses in the 10-50 nA range for about half the oocytes after 24 h. After 48 h, responses with unacylated $tRNA^{Phe}_{CUA}$ were two- to four-fold larger than the 10-30 nA signals observed with unacylated tRNA-MN3[22]. Thus we conclude that reacylation is not a

distorting factor with tRNA-MN3 and that all functioning channels contain only the desired residue at the mutation site in the α subunit.

We then studied receptors containing a variety of unnatural amino acids at positions 93, 198 and 190 (Figures 2, 4). These receptors display many characteristics similar to wild-type receptors, including responses to ACh in the micromolar concentration range and Hill coefficients near 2, suggesting that the open state of the channel is much more likely to be associated with the presence of 2 bound agonist molecules than with a single bound agonist. In addition, receptors containing unnatural amino acid residues are inhibited in a competitive fashion by dTC. Limited kinetic measurements have also been performed; voltage-jump relaxations at ACh concentrations equal to the EC50 with αTyr198(4-MeO-Phe) displayed rate constants (0.084-0.090 ms^{-1}) near that of the wild-type receptor[23]. These rate constants equal the sum of rate constants for initiating and terminating a burst of single-channel openings[24]; therefore the unnatural amino acid residues produce no major changes in the kinetics of channel gating, consistent with previous measurements on the conventional αTyr198Phe mutation[11]. Also, receptors containing unnatural side chains displayed desensitization on the same time scale as wild-type receptors.

There were, however, variations in the dose-response relation for ACh among the mutants at all the sites. For example, at 50 μM ACh the normalized responses cover a range of nearly 100-fold; and EC50 values range over nearly 10-fold.

Our results for unnatural amino acid residues at position 93 establish a prominent role for the hydroxyl group of the wild type tyrosine (Figures 2 and 4). It is surprising that all side chains with 4'-hydroxyl groups show comparable EC50 values, despite the wide range of pKa values expected for these residues (10, 9.2, 8.7, and ~5 for Tyr, 3-F-Tyr, 2-F-Tyr, and F_4-Tyr, respectively)[25]. The most straightforward explanation of this result is that all Tyr derivatives are in the same protonation state and that this state is OH, not O^-. There is ample precedent for elevated pKa values in hydrophobic protein environments[26], and apparently this is the case with the agonist binding site. In this light, 4-COOH-Phe (pKa ~ 4) can also be considered as contributing an OH group, but perhaps with suboptimal positioning. These results, in combination with the observation that 4-MeO-Phe responds no better than Phe itself, indicate that an aromatic OH at position 93 functions as a hydrogen bond donor.

The rank order of agonist EC50 for the modifications at position 198 differs markedly from that at position 93. Therefore these two tyrosines are involved in qualitatively different interactions with the agonist. The observation that 4-MeO-Phe at position 198 responds to ACh almost as effectively as Tyr at this position rules out both hydrogen bond donation and deprotonation as important characteristics of this side-chain. In addition, neither hydrophobicity scales nor any of the well known parameters that model aromatic substituent effects[27] rationalize the data for the agonist at the α198 residue, suggesting that the substituent on the aromatic ring is not the primary contributor to agonist recognition at this position. As a preliminary model, we ascribe this role to the

aromatic ring of Tyr198 interacting with the quaternary ammonium group of ACh[10, 11] through a cation-π interaction[28], as suggested for acetylcholinesterase[29]. Of course, some of the variations measured at this and the other sites could result from indirect effects of side-chain structure such as displacement of neighboring residues.

In experiments on competitive blockade[30] by dTC of receptors containing unnatural amino acids at the α198 position (Figure 2), apparent dissociation constants, K_i, range over a factor of 100. The rank order of dTC K_i values differs considerably from that for agonist EC50, amplifying and extending previous observations using conventional mutagenesis. The present data support suggestions[10,11] that the interaction between the residue at α198 and curare is predominantly hydrophobic.

At position 190, only Phe, 4-MeO-Phe, and 4-NH_2-Phe produce detectable responses; and the EC50 for these groups was more than 10-fold greater than that for the wild type receptor. Position 190 is therefore the most sensitive to structural modifications of the three positions we have studied.

The versatility in substitutions made possible by unnatural amino acid incorporation can now be applied to studies in intact cells, in particular for membrane receptors. Subtle chemical changes in the structure of an individual side chain result in readily detectable changes in the function of a large receptor/channel protein, providing decisive tests for previous hypotheses concerning ligand-receptor interactions. These initial studies have emphasized fairly conservative mutations to establish the viability of

the method. However, in cell-free systems, unnatural amino acid incorporation has been used not only to modify side chains, but also to substitute non-peptide linkages for the peptide bond, and also to incorporate fluorescent, photolabile, and spin-labeled moieties[2]. These tactics can now be applied to many questions concerning structural and functional aspects of ion channels, receptors, and transporters[31].

Notes and References

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[4]The gene for tRNA-MN3, flanked by an upstream T7 promoter and a downstream Fok I restriction site, was constructed from 8 overlapping DNA oligonucleotides and cloned into pUC19. Digestion of the resulting plasmid (pMN3) with Fok I gave a linearized DNA template corresponding to the tRNA transcript, tRNA-MN3, shown in Figure 1B minus the CA at positions 75 and 76. *In vitro* transcription of Fok I-linearized pMN3 was performed as described (J. R. Sampson and O. C. Uhlenbeck, *Proc. Natl. Acad. Sci. USA* ***85,*** 1033, [1988]). The 74-nt tRNA transcript, tRNA-MN3(-CA), was purified to single nt resolution by denaturing polyacrylamide electrophoresis and quantitated by UV absorption (J. R. Sampson, M. E. Saks, *Nucleic Acids Res.* ***21,*** 4467 [1993]). Digestion of pMN3 with Bst NI gave the template for transcription of the full-length tRNA.

[5]Most unnatural amino acids were purchased from commercial sources. For the preparation of 4-COOH-Phe, an appropriately protected Tyr (NVOC-Tyr(OH)-OtBu) was converted to the triflate (NVOC-Tyr(OTf)-OtBu) and then to the carboxylate via a Pd-catalyzed carbonylation sequence ($Pd(OAc)_2$, KOAc, dppf, CO, DMSO, 85°C, HCl quench. See: S. Cacchi, A. Lupi, *Tetrahedron Lett.* **33**, 3939 (1992)). The synthesis of dCA and the procedure for coupling the amino acid are described in ref 2.

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[16] A rabbit reticulocyte lysate system (Promega, Madison WI), was employed with incorporation of [^{35}S]-methionine.

[17]Site-directed mutagenesis was carried out using the Clontech Transformer Site-directed mutagenesis kit (Palo Alto CA). The α, β, γ, and δ subunits of AChR were subcloned into pAMV-PA, a modified pBS (S/K+) vector containing an alfalfa mosaic virus (AMV) region directly upstream from the coding region of the insert and a poly A tail downstream from the insert. The AMV region binds ribosomes tightly and increases expression (S. A. Jobling and L. Gehrke, *Nature* **325**, 622 (1987)), and we find that it is effective in both the in vitro translation and oocyte expression systems. Capped transcripts were prepared in vitro.

[18]Prior to in vitro translation or microinjection, the NVOC-aminoacyl-tRNA-MN3 was renatured by incubating at 65°C for 3 minutes. The NVOC protecting group was subsequently removed by irradiating the sample for 5 min at 23°C with a 1 kW Xenon lamp using WG-335 and UG-11 filters (Schott, Duryea PA 18642). The deprotected aminoacyl-tRNA-MN3 was immediately mixed with the desired mRNA and either added to the in vitro translation reaction or microinjected into *Xenopus* oocytes.

[19] In the δ subunit of mouse AChR, TAG is the stop codon. To prevent the aminoacylated tRNA from inserting an amino acid at this position, this sequence was mutated to TGA.

[20]Deprotected aminoacylated tRNAs were mixed with the desired AChR αTyr93TAG, αTyr190TAG, or αTyr198TAG mRNA (10:1:1:1 to 100:1:1:1) and microinjected into *Xenopus* oocytes (50 nl/oocyte; Quick, M. W., and H. A. Lester [1994], In *Ion Channels of Excitable Cells*, T. Narahashi, ed. Academic Press, pp. 261-279). Injected tRNA and mRNA concentrations were 0.4 ng/nl and 0.30 ng/nl, respectively. AChR wild type, AChR αTyr190Phe, and AChR αTyr198Phe (4:1:1:1) mRNAs were injected at concentrations of 0.035 mg/ml and 0.35 mg/ml. Electrophysiological recordings were carried out 12-24 hours after injection using a two-electrode voltage-clamp circuit. Electrode resistance was 0.5-1.0 mΩ. Bath solutions contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.5. To prevent activation of the endogenous Ca^{2+} activated Cl^- channel via muscarinic receptors, atropine (1 μM) was included in the bath solution, and Ca^{++} was omitted from the bath solution. Wild type mRNA synthesized

from the AMV vector generally gave EC50 values ~2-fold larger than those obtained with mRNA synthesized from pBluescript; we have not systematically studied this effect.

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[31]We thank V. Cornish, P. Deshpande, O. Uhlenbeck, Y. Zhang for suggestions, E. Chapman for help with synthesis, and J. Jankowski for help with the measurements. This research was sponsored by grants from the National Institutes of Health, the Office of Naval Research, the Howard Hughes Medical Institute, and the Beckman Institute at Caltech.

Figure 1A. Strategy for unnatural amino acid incorporation into membrane proteins of intact *Xenopus* oocytes. The mRNA is generated from a cDNA clone in which the codon of interest has been mutated to a nonsense codon, TAG. The unnatural side chain is denoted as R'. B. Structure of nonsense suppressor tRNA-MN3, designed to maximize suppression efficiency and to minimize reacylation.

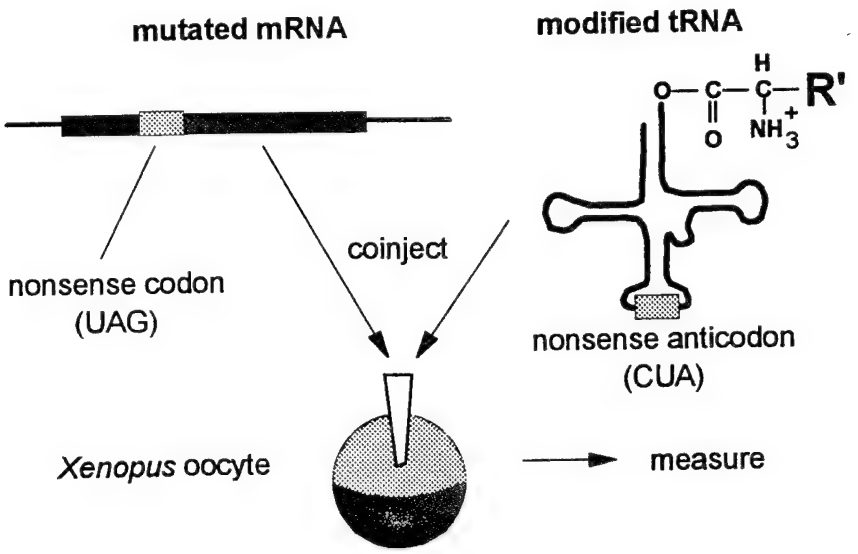
Figure 2. Natural and unnatural side chains yielding functional receptors at positions 93, 190, 198; and summary of ACh dose-response relations and dTC inhibition data for these side chains. Mean values are given for at least 6 dose-response relations (EC50) or dose-ratio analyses (K_i) from at least 2 batches of oocytes. S. E. M. ranged from 8% to 12% of the mean for EC50 values less than 600 μM and was 15-20% of the mean for higher values. NR, no detectable response; dashes, not tested.

Figure 3. Validation of the nonsense suppression technique for intact cells. (A) and (B), Reconstruction of the wild type AChR. ACh-induced currents are shown from oocytes injected with AChR β, γ, and δ subunit mRNAs plus either (A) Wild type α subunit mRNA, (B) α198TAG mRNA plus Tyr-tRNA-MN3, or (C) α198TAG mRNA plus full-length, unacylated tRNA. ACh applications are shown by the horizontal bars; concentrations in μM. (D) Validation by comparison with conventional site-directed mutagenesis. Tyrosine (the wild type residue) or phenylalanine was incorporated at positions α190 and α198. Dotted and dashed lines represent dose-response relations for the wild type AChR and for AChRαTyr198Phe obtained by conventional mutagenesis,

respectively. Data points represent the tRNA suppression technique; each point is the mean of 4-8 measurements from at least two batches of oocytes. Values for S.E.M. are smaller than the size of the symbols.

Figure 4. Normalized dose-response relations for acetylcholine receptors containing unnatural side chains at one of three positions (93, 198, or 190) in the α subunit. The symbols for the unnatural residues are consistent among the three panels; but the order of symbols in the key for each panel follows the rank order of EC50 values. The wild type residue is Tyr in each case; and the wild type dose-response relation is superimposed as a dotted line. The data for Phe at each position were obtained by both conventional mutagenesis and the nonsense suppression method (see Figure 3D) and are superimposed as dashed lines. Each symbol represents measurements on at least 5 oocytes from at least 2 separate batches Individual dose-response relations were fit to the Hill equation, $I/I_{\max} = 1/\left(1 + \{\mathrm{EC50}/[A]\}^{n_H}\right)$ where I is the current for agonist concentration $[A]$, I_{max} is the maximum current, EC50 is the concentration to elicit a half-maximum response, and n_H is the Hill coefficient. Values for n_H ranged from 1.6 to 1.8. I_{max} values exceeded 500 nA in all cases except 4-NH_2-Phe at position 190, where I_{max} was ~ 100 nA. Values for S.E.M. are shown where they exceed the size of the symbols.

A. Unnatural Amino Acid Incorporation in Oocytes

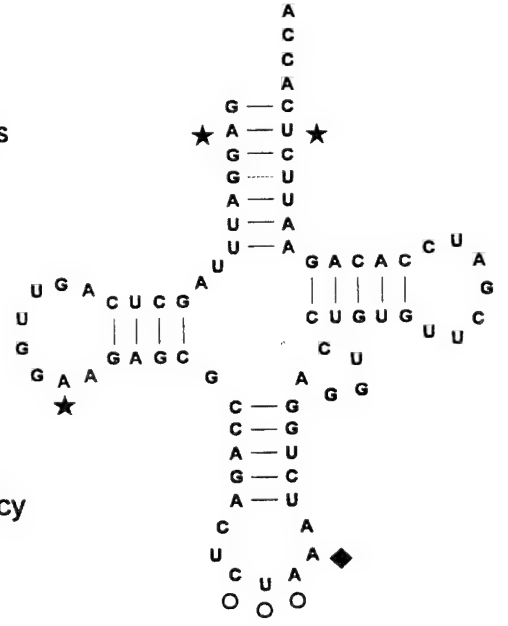


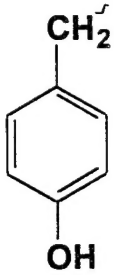
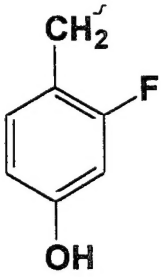
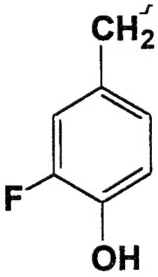
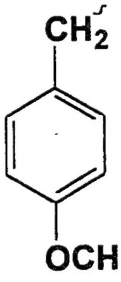
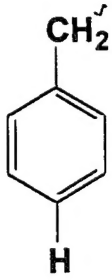
B. Nonsense Suppressor Based on Yeast $tRNA^{Phe}$

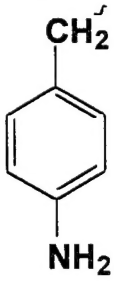
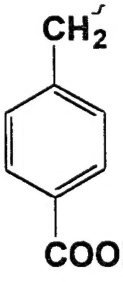
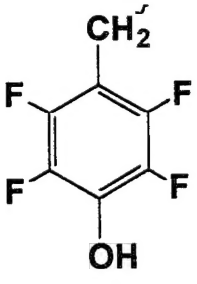
★ changed to suppress reacylation

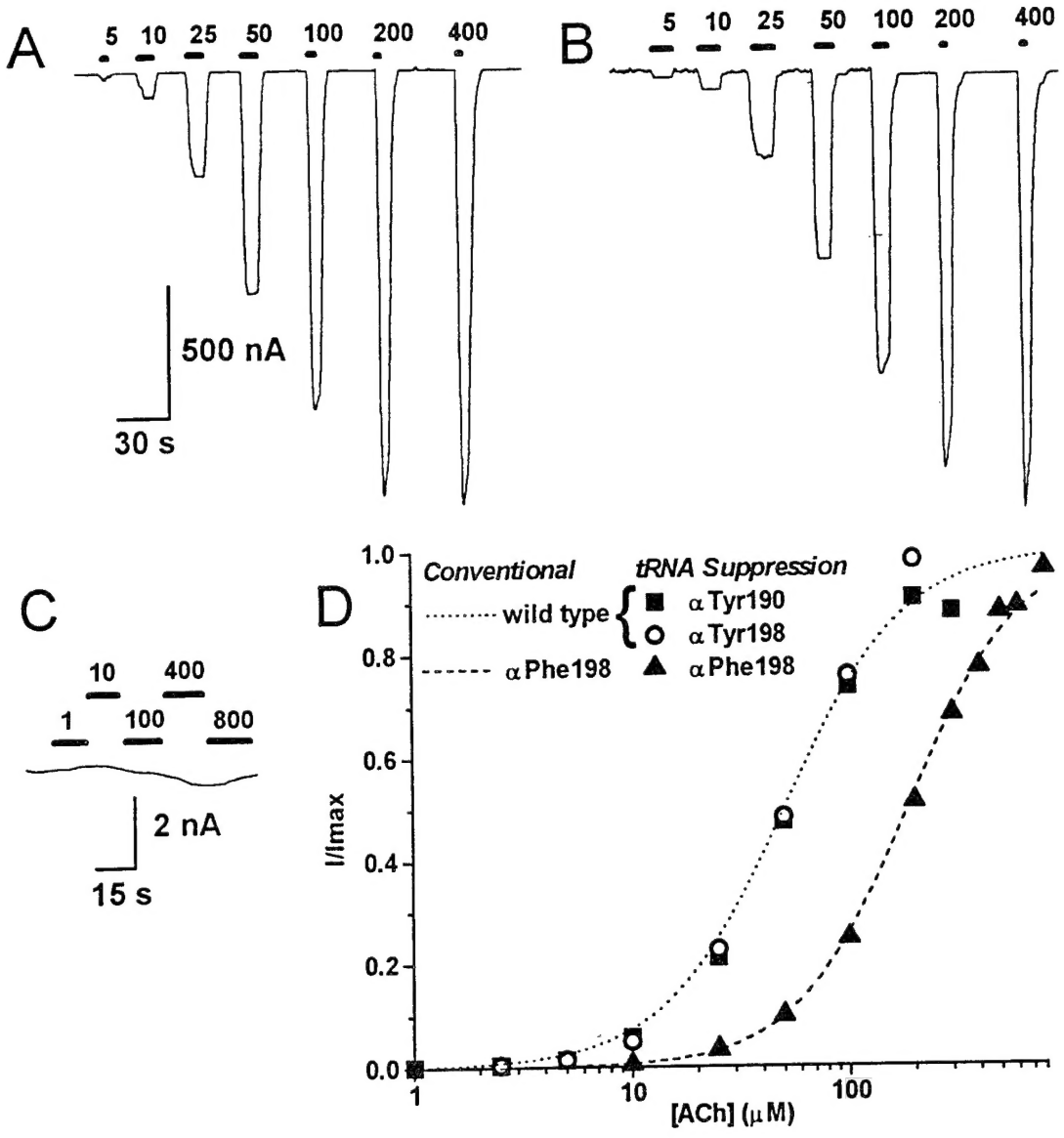
◆ changed to increase suppression efficiency

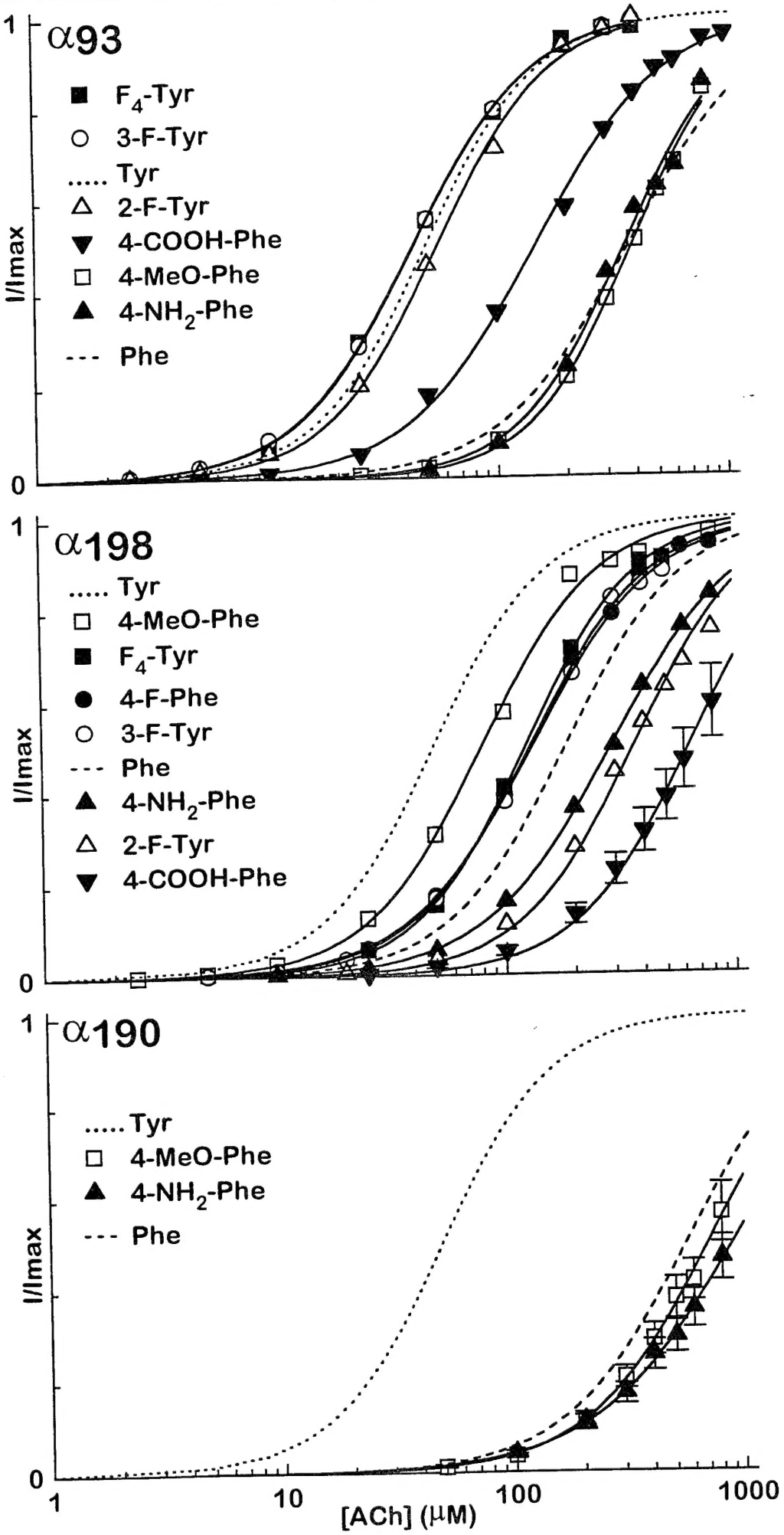
○ anticodon



						
		Tyr	**2-F-Tyr**	**3-F-Tyr**	**4-MeO-Phe**	**Phe**
EC50	*93*	49	44	38	388	429
for ACh	*190*	49	NR	NR	704	566
(μM)	*198*	49	348	144	84	215
dTC K_i (nM)		37	32	37	8.6	2.3

					
		4-NH_2-Phe	**4-F-Phe**	**4-COOH-Phe**	**F_4-Tyr**
EC50	*93*	364	-	149	44
for ACh	*190*	904	NR	NR	NR
(μM)	*198*	278	143	669	134
dTC K_i (nM)		12	3.6	240	33


A
5 10 25 50 100 200 400
500 nA
30 s
B
5 10 25 50 100 200 400
C
10 400
1 100 800
2 nA
15 s
D
Conventional
tRNA Suppression
wild type
αTyr190
αTyr198
αPhe198
αPhe198
I/Imax
1.0
0.8
0.6
0.4
0.2
0.0
1
10
100
[ACh] (μM)


α93
F4-Tyr
3-F-Tyr
Tyr
2-F-Tyr
4-COOH-Phe
4-MeO-Phe
4-NH2-Phe
Phe
I/Imax
1
0
α198
Tyr
4-MeO-Phe
F4-Tyr
4-F-Phe
3-F-Tyr
Phe
4-NH2-Phe
2-F-Tyr
4-COOH-Phe
I/Imax
1
0
α190
Tyr
4-MeO-Phe
4-NH2-Phe
Phe
I/Imax
1
0
1
10
100
1000
[ACh] (μM)

OFFICE OF THE UNDER SECRETARY OF DEFENSE (ACQUISITION)
DEFENSE TECHNICAL INFORMATION CENTER
CAMERON STATION
ALEXANDRIA, VIRGINIA 22304-6145

IN REPLY REFER TO DTIC-OCC

SUBJECT: Distribution Statements on Technical Documents

TO: OFFICE OF NAVAL RESEARCH
CORPORATE PROGRAMS DIVISION
ONR 353
800 NORTH QUINCY STREET
ARLINGTON, VA 22217-5660

1995 1027 024

1. Reference: DoD Directive 5230.24, Distribution Statements on Technical Documents, 18 Mar 87.

2. The Defense Technical Information Center received the enclosed report (referenced below) which is not marked in accordance with the above reference.

FINAL REPORT
N00014-91-J-1231
TITLE: NICOTINIC RECEPTOR BINDING
SITE PROBED WITH UNNATURAL
AMINO ACID INCORPORATION IN
INTACT CELLS

3. We request the appropriate distribution statement be assigned and the report returned to DTIC within 5 working days.

4. Approved distribution statements are listed on the reverse of this letter. If you have any questions regarding these statements, call DTIC's Cataloging Branch, (703) 274-6837.

FOR THE ADMINISTRATOR:

1 Encl

GOPALAKRISHNAN NAIR
Chief, Cataloging Branch

FL-171
Jul 93